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Research Article

Comparison of three strategies for local bioassessments in streams using environmental DNA and RNA metabarcoding of macroinvertebrates

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Abstract

Environmental DNA (eDNA) metabarcoding of streams can deliver information on biological communities with minimally invasive efforts and at low costs. However, the captured eDNA may not necessarily originate only from organisms physically present at a local site but also come via drift from sources upstream. Therefore, data on community composition can be inherently different to specimens occurring at a certain site, limiting direct comparisons to site-based assessment data. To increase the local signal, two possible ways were recently proposed: First, targeting eRNA collected from water samples, which degrades faster than eDNA, and second, using eDNA from water, in which organisms from the stream were shortly incubated in a water container. Based on these two options, we here derived and tested three strategies to obtain a more localized signal of biodiversity: 1. eRNA metabarcoding of a direct stream water sample, 2. eDNA and eRNA metabarcoding of water, in which animals collected via kicknet multi-habitat sampling were shortly incubated, and 3. eDNA and eRNA metabarcoding of water, in which natural substrate exposures (NSEs) that were actively colonised by invertebrates in the stream for four weeks were shortly incubated. For comparison, we used eDNA directly isolated from the stream as the more regional signal. As the local point-based sample reference, we used the animal bulk sample that was incubated. While our results support that eRNA collected from the stream (strategy 1) does not improve the local signal, both incubation strategies increased the proportion of species identified by both metabarcoding of eDNA/eRNA and the locally collected bulk sample by about 20%. Interestingly, we detected 20% more species in the NSEs (strategy 3) than in the kicknet samples (strategy 2), highlighting the potential of this less invasive invertebrate sampling approach. In summary, our study demonstrates that incubating collected animals in stream water before filtering the water increases the local community signal and provides an animal-friendly strategy for biomonitoring.

Key words: eDNA, eRNA, freshwater, local bioassessment, macroinvertebrates, metabarcoding

Introduction

In view of the increasing environmental pressures that we are currently facing, there is a growing demand to minimise the impact on nature when assessing the status of freshwater habitats. This necessitates a shift in focus towards non-invasive methods, especially when assessing the status of heavily impacted or restored freshwater habitats. Thus, considering the use of environmental DNA (eDNA)-based approaches in bioassessment and monitoring is increasingly being asked for, since they also offer a combination of high taxonomic resolution, easy and fast sampling, and the potential for a more holistic sampling. Also, the number of published studies highlighting the value of eDNA-based metabarcoding for freshwater biomonitoring is increasing continuously (Pawlowski et al. 2018 and see references therein). A key reason for this development is the efficiency of the method, as biodiversity can be assessed rather holistically and non-invasively on the basis of a single water sample (Deiner et al. 2016; Stat et al. 2017; Macher et al. 2021a).

However, metabarcoding based on water samples from streams is also subject to numerous uncertainties, including DNA retention and re-suspension from the sediment as well as the distribution of DNA molecules in the water column which are influenced by a stream's hydromorphology (Deiner and Altermatt 2014; Shogren et al. 2017; Wacker et al. 2019). The imprecision in the sources and composition of the eDNA signal can be a problem as it may skew bioassessment results and make them less comparable to local assessments like tissue DNA metabarcoding that rely on organisms physically collected at a specific site (Dejean et al. 2011; Thomsen et al. 2012a, 2012b; Pilliod et al. 2014).

One key group in freshwater biomonitoring, which is collected physically at a site for routine biomonitoring, are benthic invertebrates. Being sensitive to environmental stressors, they are employed as biological quality elements (BQE) to evaluate the condition of lotic and lentic water bodies in many countries and to apply counter measures (e.g., Water Framework Directive, US Clean Water Act; see Buss et al. 2015 for an overview). However, eDNA-based metabarcoding fails to comprehensively capture the local invertebrate community compared to tissue-based metabarcoding (Macher et al. 2018; Hajibabaei et al. 2019; Gleason et al. 2021; Pereira da Conceicoa et al. 2021; Múrria et al. 2024). Accordingly, methods that require the killing of organisms, such as for detailed morphological identification or bulk metabarcoding, remain the preferred methods when the objective is to obtain local results (but see Li et al. 2021 for a comparison to eDNA metabarcoding).

Recently, two different strategies have been proposed to advance genetic bioassessments at specific sites based on water samples: analysis of eRNA metabarcoding (Pochon et al. 2017; Pawlowski et al. 2018; Cristescu 2019; Yates et al. 2021) and analysis of eDNA/eRNA of captured aquatic invertebrates incubated in water for a short period (Sander et al. 2025).

eRNA metabarcoding: In contrast to eDNA, eRNA has a higher production and turnover rate (Eigner et al. 1961; Mengoni et al. 2005), suggesting that most of the captured eRNA was recently released to the environment from living animals present at a sampling site (Pochon et al. 2017; Marshall et al. 2021) (see Fig. 1A for a schematic depiction). However, recent evidence suggests that the situation is more complex than first expected. Studies comparing the decay

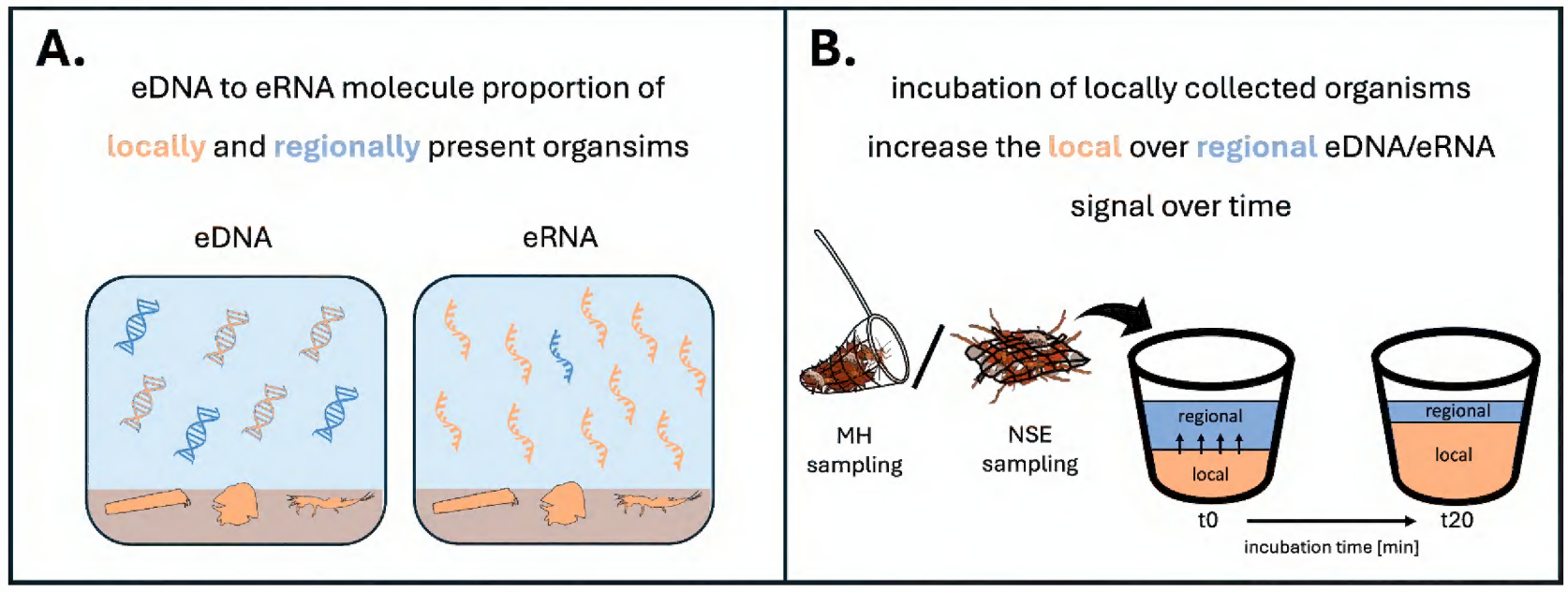

Figure 1. Schematic depiction of the underlying assumptions of the three strategies. **A.** Depicts the differences in the proportion of the local (from living animals present at a site) and regional (from animals living further upstream of the site) molecules between eDNA (left) and eRNA (right) (strategy 1: metabarcoding of eRNA obtained from a water sample taken directly from the stream); **B.** Depicts the enrichment of DNA from local organisms with time in a water container filled with stream water after incubating the animals captured from the stream using either MH sampling (strategy 2: metabarcoding of eDNA/eRNA obtained from locally sampled organisms (MH) incubated in water) or NSE sampling (strategy 3: metabarcoding of eDNA/eRNA obtained from locally colonised NSEs incubated in water).

rates of eRNA and eDNA in water samples reported a similar decay rate for both types of molecules (Wood et al. 2020; Marshall et al. 2021) and the binding of eDNA and eRNA molecules to an organic matrix, such as biofilm, or the presence within shed cells, can further enhance the stability of these molecules, thereby increasing their detectability to up to 21 days (Wood et al. 2020; Jo 2024). It is important to consider the different factors influencing eRNA release and decay in the water column, and which RNA type (mRNA vs. rRNA) and gene region to choose for eRNA metabarcoding. There can be considerable differences in the number of RNA copies retained from different gene regions of the same RNA type as the genes release different amounts of RNA molecules (Sidova et al. 2015; Marshall et al. 2021; Jo et al. 2023). There are only a few studies which assessed the potential of eRNA metabarcoding. Here, eRNA-based metabarcoding for vertebrates shows a stronger local signal than eDNA-based metabarcoding (Macher et al. 2024; Zhang et al. 2024). For invertebrates eRNA-based analysis leads to a lower number of detected species of invertebrates and a higher positive predictability but a less reliable detection of the sensitivity to environmental variables (Miyata et al. 2022; Macher et al. 2024; Zhang et al. 2024).

Incubation strategy: The release and persistence of eDNA from a variety of vertebrate and invertebrate species has been the subject of numerous studies, which found an increase in released DNA molecules after a short incubation in a water-filled container, ranging from minutes to hours (Andruszkiewicz Allan et al. 2021; Sander et al. 2025). Sander et al. (2025) expanded this incubation strategy on a bulk stream sample comprising an unknown composition of benthic invertebrates. The aim was to improve the detection of the local invertebrate community by incubating the animals from the locally collected bulk sample for a certain time and analysing the enriched water sample from the incubation medium (see Fig. 1B for a schematic depiction). Here, the proportion of species detected using the enriched eDNA from the incubation medium compared to metabarcoding of the locally collected bulk sample reached immediately 60% after transferring the animals to the incubation medium (Sander et al. 2025). The results highlight the promise

of the incubation strategy for a more local assessment of biological communities using a less invasive approach, because the animals can be released back to the stream. However, the sampling was conducted using multi-habitat (MH) kicknet samples, in accordance with the national requirements of the Water Framework Directive (WFD). This sampling approach yields reliable data but the use of kicknet sampling is generally rather destructive to the streambed and stressful for the sampled organisms compared to simple eDNA stream water sampling.

For this study, we therefore propose a third strategy, in which natural substrate exposures (NSEs), i.e., pre-compiled substrates, similar to Autonomous Reef Monitoring Structures (ARMS) applied in marine biodiversity monitoring (Leray and Knowlton 2015; Ransome et al. 2017), are used to sample the stream organisms. NSEs can be inserted into the stream for a time period of a few weeks and colonised by invertebrates and subsequently be recovered without a time-consuming and more invasive bottom habitat sampling (Dumeier et al. 2018). A first marine case study from Nichols et al. (2022) found that taxa lists obtained from incubating NSEs in a water-filled container before eDNA sampling differed substantially from those obtained from the tissue of the sampled organisms. However, they only filtered 0.2% of the incubation medium. The simplicity and non-invasiveness of the NSE sampling approach renders it an optimal sampling method for combining it with the incubation strategy proposed by Sander et al. (2025). The functionality of NSEs for freshwater systems was tested in studies by Dumeier et al. (2018, 2020), where the authors successfully relocated macroinvertebrates via pre-compiled substrates. Hensel and Kiel (2012) reported a mortality rate below 1% and Meyer (2017) indicated a 97% survival rate for EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa after the removal of NSEs. Compared to kicknet sampling, the approach is thus less invasive for animals. However, the workload per sampling spot is greatly increased in comparison to kicknet sampling if the same stream area is sampled. Therefore, we used for our study a smaller sampling area for the NSE sampling approach in comparison to the MH sampling approach. Further, less habitats are sampled using NSEs than with the MH sampling approach via kicknet sampling, because the latter includes the sampling of small invertebrates colonising the sediment and which may not use the provided NSE substrates as their habitat.

In view of the inherent challenge to obtain more localised bioassessment data in streams using metabarcoding of water samples, we evaluated the performance of the three above introduced strategies:

1. Metabarcoding of eRNA obtained from a water sample taken directly from the stream;
2. Metabarcoding of eDNA/eRNA obtained from organisms collected locally using multi-habitat (MH) kicknet sampling after 20 min incubation in water;
3. Metabarcoding of eDNA/eRNA obtained from locally colonised natural substrate exposures (NSEs) after 20 min incubation in water.

As a benchmark we used 1. taxa lists obtained via metabarcoding of locally sampled bulk samples (MH or NSE) as our local signal reference and 2. taxa lists obtained via eDNA metabarcoding of a water sample directly taken from a stream as our regional signal reference. Then we compared the resulting taxa lists of the benchmark to the taxa lists obtained via the three strategies outlined above.

In view of the findings of previous studies outlined above, we hypothesise that:

1. species lists obtained via eRNA metabarcoding of water samples show a greater overlap with the locally collected MH and NSE bulk samples than those obtained via eDNA metabarcoding of stream water. The higher overlap for eRNA metabarcoding is predicted given that eRNA originates mostly from local, living organisms (Fig. 1A, strategy 1)

2a. species lists obtained via eDNA/eRNA metabarcoding of water, in which animals (MH, strategy 2) or substrates (NSE, strategy 3) were shortly incubated, show a greater overlap with the locally collected MH and NSE bulk samples than eDNA metabarcoding of a stream water sample. This is due to the gradual increase in the number of DNA/RNA molecules shed from living organisms into the water, changing the ratio between eDNA/eRNA from organisms living upstream and eDNA/eRNA shed from the living organisms in the incubated bulk sample (Fig. 1B, strategy 2 and 3).

2b. species lists obtained via eRNA metabarcoding of the incubated water (MH, strategy 2; NSE, strategy 3) show a slightly greater overlap with the locally collected MH and NSE bulk samples than eDNA metabarcoding of the incubated water (Fig. 1A, strategy 1, see hypothesis 1).

3. between the two incubation-based strategies, the one based on multi-habitat sampling (strategy 2) detects more species than the natural substrate exposures (strategy 3). The higher number of species detected using multi-habitat sampling is predicted due to i) the broader range of habitats it covers and ii) the larger area being sampled, ultimately resulting in a higher recovery rate of rare or habitat-specific species.

The ultimate aim of the study was to derive recommendations for minimally-invasive yet local bioassessment using eDNA/eRNA metabarcoding.

Methods

Preparation of natural substrate exposures (NSEs)

We employed two types of natural substrate exposures (NSEs), wood-leaf mix and gravel, to cover the majority of habitats present in the stream. Leaves, wood and gravel were sampled directly at each sampling site to preserve the natural environments of the streams and prevent contamination by pesticides and pathogens between different streams. NSEs were created following the protocol used by Dumeier et al. (2018). NSEs consisted of 30 cm x 30 cm Nylon nets (Schutznetze24 GmbH, Aßlar-Berghausen, Germany) with a mesh size of 1 cm. Leaves, wooden sticks and gravel were dried at room temperature for one day separately for each stream. For the gravel NSEs, we used 2100 g gravel with a diameter of > 1 cm to prevent the material from being flushed out of the nets (see Suppl. material 1: fig. S1A). The wood-leaf mixture consisted of 105 g wooden sticks cut to a length of 20 cm (diameter: 0.5–2 cm) tied together in three bundles and 20 g leaves per NSE (see Suppl. material 1: fig. S1B). After weighing, the leaves were soaked in water to prevent crumbling while transferring them to the nylon net. In total, we prepared 32 NSEs with four NSEs per sample spot split into two wood-leaf mix NSEs and two gravel NSEs (Fig. 2).

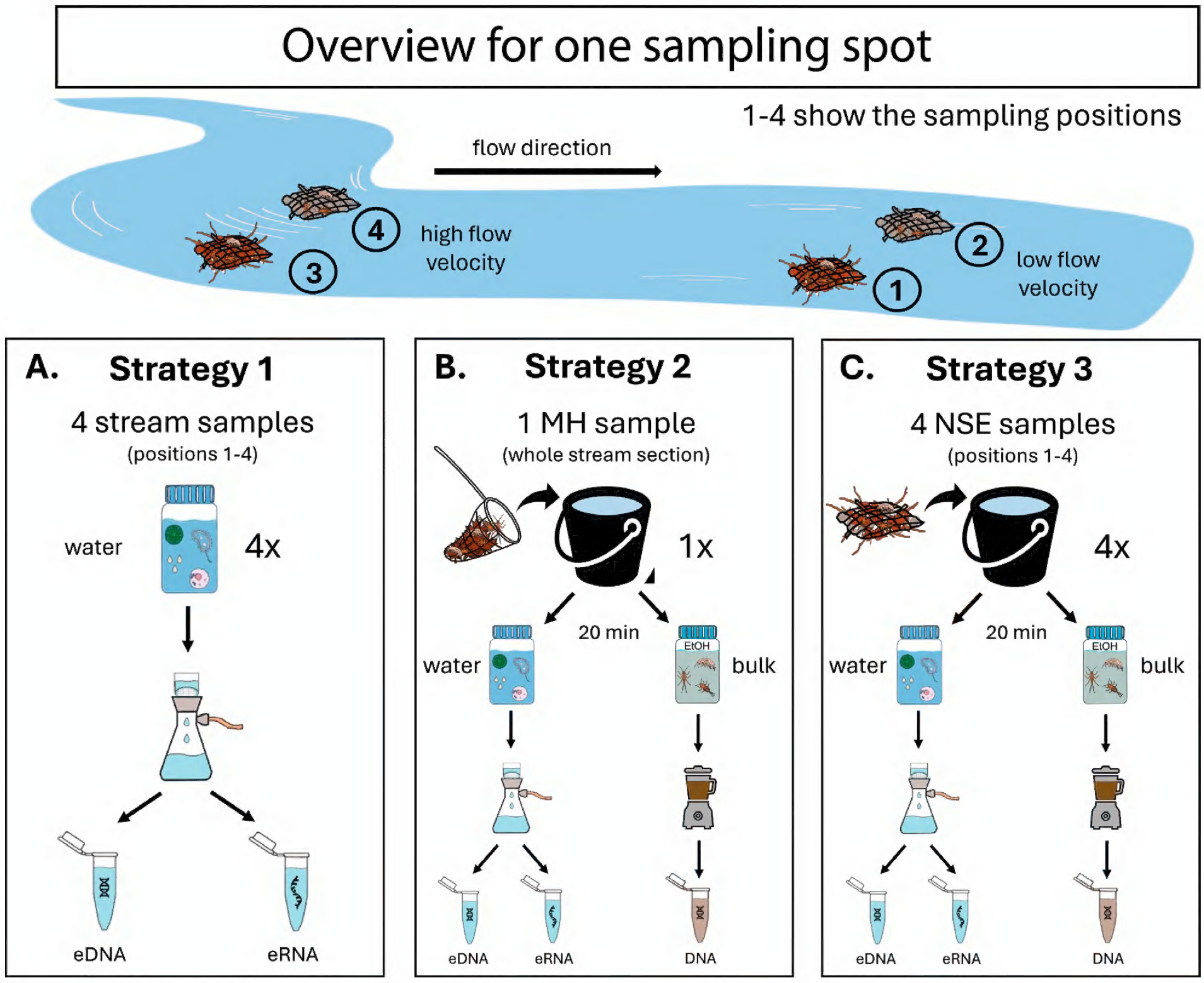

Figure 2. Overview of the sampling, sample handling and DNA and RNA extraction approaches from the different samples for the three tested strategies to improve local signals (A-C). We sampled four streams: Emscher, Ennepe, Felderbach, Grüner Bach in Germany each at two sampling spots with four sampling positions for the stream water and NSE samples (32 sampling positions in total). For the MH samples, the whole stream section per sampling spot was sampled (8 stream sections in total reflecting the different microhabitats). A: Sampling approach to test strategy 1 (stream water samples for eDNA/eRNA metabarcoding); B: Sampling approach to test strategy 2 (incubation of MH samples and comparison of eDNA/eRNA enriched in water to bulk MH DNA metabarcoding); C: Sampling approach to test strategy 3 (incubation of NSEs and comparison of eDNA/eRNA enriched in water to bulk NSE DNA metabarcoding). In total we analyzed 72 eDNA samples (32 stream eDNA, 32 NSE eDNA, 8 MH eDNA), 144 eRNA samples (64 stream eRNA (32 DNase digested, 32 not DNase digested), 64 NSE eRNA (32 DNase digested, 32 not DNase digested), 16 MH eRNA (8 DNase digested, 8 not DNase digested)) and 40 bulk DNA samples (32 NSE bulk, 8 MH bulk).

Sampling and experimental setup

We sampled four streams at two spots each: Emscher, Ennepe (spot 1: 51°16'59"N, 7°23'11"E, spot 2: 51°16'49"N, 7°23'25"E), Felderbach (spot 1: 51°21'11"N, 7°10'13"E, spot 2: 51°20'55"N, 7°10'19"E) and Grüner Bach (spot 1: 51°19'49"N, 7°40'51"E, spot 2: 51°19'49"N, 7°41'02"E) which are all located in North-Rhine Westphalia, Germany (see Suppl. material 1: fig. S2). The sampling locations along each stream were a minimum of 500 m apart. For the Emscher, we sampled the tributaries Boye (spot 1: 51°33'40"N, 6°55'59"E) and Borbecker Mühlenbach (spot 2: 51°26'16"N, 6°58'03"E) since the Emscher itself was too

large and deep, with a high current, to safely and stably attach substrates to the riverbed. All streams, except the two Emscher tributaries, were stream type 5 (coarse stony substrates of high silicate concentration) and featured a gravel riverbed. The riverbed of the Borbecker Mühlenbach (stream type 6: small fine substrate dominated calcareous highland rivers) also consisted mostly of gravel and the Boye (stream type 14: small sand-dominated lowland rivers) had large parts of sand covering the riverbed (LAWA-Typology, Dahm et al. 2014). In April 2023, the NSEs were secured to the riverbed using steel rods and tent pegs to ensure stability (see Suppl. material 1: fig. S3). One NSE of each type was positioned in a low-flow section (pool) and the other in a high-flow section (riffle) to capture a diverse range of species, as species richness, identity and the proportion of feeding types can differ between pools and riffles (Cummins 2016). The NSEs were left in the streams for four weeks to allow colonisation by the local macroinvertebrate community. Dumeier et al. (2018) found that a six-week settlement period resulted in the highest numbers of species and specimens. However, we opted for a four-week settlement period due to the rapid breakdown of leaves by shredders, which would ultimately leave no remaining food source to attract macroinvertebrates. Two weeks following the placement, a visual inspection was conducted to verify that each NSE had remained securely positioned in the streambed and was still immersed in stream water. All eight NSEs at Ennepe had to be relocated, along with two at Felderbach, due to decreased water levels which left the NSEs only half submerged in the stream. The respective NSEs were moved to a slightly deeper point a few centimetres from their original position. For the Boye, one NSE had to be freed from sand covering it.

After the four-week settlement of NSEs in May, we sampled each spot using three different approaches adjusted to our three strategies (Fig. 2). First, we took four 500 mL direct water samples from the stream located in proximity downstream to the NSEs to prevent contamination of the NSE samples (Fig. 2A.). Samples were immediately filtered afterwards in the field using a vacuum pump and 0.45 µm cellulose nitrate membrane filters (diameter: 47 mm; Nalgene, New York, USA) and stored on ice. These samples are hereafter referred to as stream water samples.

Second, we carefully sampled the NSEs from the stream with a sampling net to avoid losing macroinvertebrates that inhabited the NSEs. Next, we transferred the NSEs from the net to a 20 L bucket filled with 10 L stream water. After 15 min of incubation, the water in the bucket was stirred for 60 s to mix the water as well as the DNA in it. Due to the water's high turbidity, we waited four minutes to let the suspended particles settle down to reduce the risk of filter clogging. After a total incubation of 20 min, 500 mL water from the bucket was filtered using a vacuum pump and 0.45 µm cellulose nitrate membrane filters (diameter: 47 mm; Nalgene, New York, USA) and stored on ice (NSE eDNA samples). The animals including debris were preserved in 96% technical ethanol after removing larger sticks and gravel (NSE bulk samples) (Fig. 2C.).

Third, we sampled a transect of the stream using multi-habitat kicknet sampling following the protocol used in Meier et al. (2006). We took ten instead of 20 subsamples per stream due to their smaller size and low habitat variations (see Suppl. material 2 for information on the different habitat structures). The whole kicknet sample was transferred to a 20 L bucket filled with 10 L stream water. From here, the sampling procedure was identical to the one used for the NSEs including volume of water filtered and animal preservation (Fig. 2B).

The water samples from the bucket are hereafter referred to as MH eDNA samples and the samples including the animals and debris as MH bulk samples. Between each sample and sampling approach, gloves were changed and the sampling net washed with stream water. We used negative filter controls ('field blanks') at each sampling site to check for air-contamination. For this, only the surrounding air was filtered using a vacuum pump. All water samples were immediately stored on ice at the field before and after filtration and later stored in a laboratory freezer at -80 °C. The preservative of the organismal bulk samples was changed after 24 h with fresh 96% technical ethanol and samples were stored afterwards at room temperature. In total, we sampled 72 water samples (9 per sampling spot), 40 bulk samples (5 per sampling spot) and 8 air-contamination controls (1 per sampling spot).

Lysis and extraction of DNA and RNA

Bulk samples were homogenised and extracted following the steps used in Sander et al. (2025). For homogenisation of the bulk samples, the protocol from Buchner et al. (2021a) was used. Bulk samples were homogenised unsorted using a kitchen blender (Mini Blender & Blender Smoothie, Homgeek, China) and 1 mL of the homogenate per sample was transferred to a twist-top tube with one spoon of each 1 and 2 mm zirconia beads. For lysis of the bulk samples, samples were centrifuged for 3 min at maximum speed, and the supernatant (ethanol) was discarded. Then, 900 µL TNES and 100 µL Proteinase K were added to each sample and the samples were subsequently ground for 2 min at 2400 rpm and then lysed at 65 °C for 20 min. For extraction, we followed the protocol from Buchner (2022a) using a vacuum manifold. The following changes were done to the protocol: 500 µL binding buffer and 250 µL lysate per sample were mixed at 900 rpm for 5 min and 750 µL of the mixture of each sample was transferred to a 96-well filter plate placed in a vacuum manifold. Subsequently samples were washed twice with 500 µL wash buffer. The following steps were done according to the protocol. Extraction was performed in replicates, and we included six extraction negative controls per plate, which were positioned on the 96-well plate as described in Elbrecht and Steinke (2019). Afterwards, the extracted DNA was purified to avoid inhibition during PCR. For this, we mixed 80 µL Sera Speed beads (carboxylated 1:50 in SPRI buffer) with 40 µL of the extract per sample. Samples were incubated for 5 min at 1000 rpm on a thermomixer and then placed on a magnet for 2 min, after which the supernatant was discarded. We washed the samples twice with 100 µL wash buffer and dried them for 5 min to remove excess ethanol. After removing the magnet, samples were eluted with 40 µL elution buffer and mixed for 5 min at 1000 rpm. Then, the samples were again placed on the magnet for 2 min to settle the beads and 35 µL of the supernatant were transferred to a new 96-well plate. For co-extracting RNA and DNA from the filters, we used the protocol from Buchner (2023). For this, the filters were chopped into small pieces before lysis in a sterile eDNA laboratory. The following changes were made to the protocol for filter samples: for sample preparation, we used 1000 µL GITC lysis buffer (4 M guanidinium thiocyanate, 10 mM Tris) for each sample and lysate clearing and pre-filtering were done using 650 µL of the crude lysate for each filter column. We used extraction replicates for each filter and

therefore split the crude lysate to 300 µL each and transferred them to a new plate. For RNA precipitation and binding, we used 300 µL 70% ethanol. All remaining steps were done following the protocol (Buchner 2023). For the filter extraction we included 11 extraction negative controls adapted to the number of samples per plate and positioned according to Elbrecht and Steinke (2019). The lysate of the 8 air-contamination controls was extracted and amplified for the eDNA and eRNA workflow (with and without DNase digestion) resulting in a total of 24 air-contamination control samples (without counting replicates).

DNase digestion and reverse transcription

For DNase treatment, 9 µL of extracted eRNA were mixed with 0.5 µL of 10 x ezDNase Buffer and 0.5 µL of ezDNase enzyme (ThermoFisher Scientific, Germany) and the mixture incubated for 2 min at 37 °C. For reverse transcription of RNA to cDNA, 1 µL of Superscript IV VILO Master mix (ThermoFisher Scientific, Germany) was added to each sample as well as 9 µL of PCR-grade water to get a total volume of 20 µL per reaction. Each reaction was thoroughly mixed and incubated for 10 min at 25 °C followed by 10 min at 50 °C and finally for 5 min at 85 °C. Afterwards the reactions were stored at -20 °C until PCR amplification.

PCR and sample preparation

We used the primer pair fwhF2/fwhR2n targeting the COI gene region for metabarcoding of DNA and RNA, which was designed for freshwater invertebrates (Vamos et al. 2017). We performed a two-step PCR approach with tagged versions of the fwhF2/fwhR2n primer pair with varying length and a universal tail in the first step (Leese et al. 2021). We did not use additional PCR replicates and analysed the extraction replicates separately until after sequencing. For the first step, each reaction contained 1x Multiplex PCR Master Mix (Qiagen Multiplex PCR Plus Kit), 0.2 µM of each primer, 0.5x CoralLoad Dye and 1 µL purified DNA extract, which was filled up to a total of 10 µL with PCR-grade water. The cycling conditions consisted of the following steps: 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 58 °C for 90 s, 72 °C for 30 s, with a final elongation at 68 °C for 10 min. Afterwards, the samples were purified to reduce potential primer-dimers that were formed during the first step, following the PCR cleanup and size selection with a magnetic beads protocol available at Protocols.io (Buchner 2022b). The second PCR step used primers matching the universal tail which included one of 96 i5/i7 indices and the P5/P7 Illumina adapter (Buchner et al. 2021a, 2021b). The reactions of the second PCR contained the following ingredients: 1x Multiplex PCR Master Mix (Qiagen Multiplex PCR Plus Kit), 0.5 µM of each primer, 0.5x CoralLoad Dye and 2 µL of purified PCR product. The cycling conditions consisted of the following steps: 95 °C for 5 min, 25 cycles of 95 °C for 30 s, 61 °C for 90 s, 72 °C for 30 s, and a final elongation of 68 °C for 10 min. Afterwards PCR products were normalized with magnetic beads (Buchner 2022c), pooled, purified and their DNA concentrations measured using a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies GmbH, Germany) with the NGS Standard Sensitivity Kit. To remove PCR bubbles from our library, which potentially formed after PCR, we performed a reconditioning PCR (Buchner 2024). In short, we concentrated our library, performed the reconditioning PCR,

purified the PCR product, did a size-selection to remove potential primer-dimers and did a last quality control using a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies GmbH, Germany). The final library was sent to Macrogen Europe B.V. (Amsterdam, Netherlands) for sequencing on a single flow cell using an Illumina NovaSeq 6000 sequencer (150 bp paired-end reads, S4 flow cell) and 5% Phi-X spike-in.

Data analysis

We used the APSCALE pipeline version 1.6.3 (Buchner et al. 2022), which is based on VSEARCH and cutadapt (Martin 2011; Rognes et al. 2016), with standard settings to process the demultiplexed sequences. OTUs were clustered with a 97% similarity threshold and taxonomically assigned via BOLDigger version 2.1.1 to a sequence deposited in BOLD (Buchner and Leese 2020) with the following thresholds: ≥ 98% for species level, ≥ 95% for genus level, ≥ 90% for family level, ≥ 85% for order level, < 85% for class level. These are the default settings in BOLDigger where the species threshold is based on the study of Hebert et al. (2003). Afterwards, the extraction replicates were merged, retaining all OTUs from both replicates. Then, the maximum number of reads among negative controls per OTU was subtracted from each OTU in the samples to account for contamination and tag switching. OTUs with an assignment to at least family level were retained for further analysis. Lastly, the dataset was filtered for macroinvertebrates and all terrestrial taxa were removed.

Statistical analysis

All statistical analyses and plots were generated with R version 4.0.5 (R Core Team 2021) and its implemented stat package and the ggplot2 package (Wickham 2011). Given the discrepancy in the number of MH and NSE samples (one sample versus four samples) collected from each site, we subsampled the MH bulk and eDNA samples to 350,000 reads per sample, while each of the four NSE bulk and eDNA samples per site were subsampled to 87,500 reads in order to ensure the comparability of the results and if species lists and the number of species shared between sampling approaches obtained before and after subsampling reads would differ. We used the function rarefy implemented in the R-package vegan (Oksanen et al. 2020) for subsampling each of the samples to the desired read numbers. Venn diagrams were created using TaxonTableTools version 1.4.8 (Macher et al. 2021b). We tested if there is a significant difference in the number of species, OTUs and reads shared between eDNA/eRNA and bulk DNA metabarcoding detected between the different sampling approaches (stream water sampling, MH incubation, NSE incubation). For this, we checked if the samples were normally distributed using a manual inspection based on Q-Q plots and a Shapiro-Wilk test. Additionally, we tested the samples for homogeneity with a Levene test using the R-package car version 3.1-1 (Fox and Weisberg 2019). For species and OTUs, samples were normally distributed and showed no heterogeneity in their variance. Therefore, an ANOVA (ANalysis Of VAriance) was performed (Suppl. material 3). For the number of reads, the samples were not normally distributed, and we therefore performed a Kruskal-Wallis test. To see which sampling approaches were significantly different from

each other, we did pairwise comparisons using a pairwise t-test with a False Discovery Rate (FDR) p-value correction for species and OTUs and for reads a pairwise Wilcoxon rank-sum test with an FDR p-value correction. To visualise differences between the species community composition of the different sampling approaches and the two sample types bulk and eDNA, non-metric multidimensional scaling (NMDS) based on Jaccard's distance was conducted using the R-package vegan. An ANOSIM test using Jaccard's distance and a pairwise comparison using the R-package pairwiseAdonis (Martinez Arbizu 2020) was used to test for differences in the species community composition between the three sampling approaches used (stream water sampling, MH incubation, NSE incubation). Further, we tested if samples would differ between streams.

Results

Metabarcoding analysis of all sample types (eDNA, eRNA, bulk) combined produced 334,260,739 reads that clustered into 23,381 OTUs after quality filtering (see Suppl. material 4). Read numbers per sample ranged from 74,499 to 2,242,111 reads, with a mean read number of 1,294,049 (see Suppl. material 1: table S1 for details on the different sample types). The negative controls contained 659,594 reads (0.22% of the total reads), with one replicate of one of the 63 negative controls (24 air-contamination controls + 39 extraction negative controls), positioned on the bulk metabarcoding plate, containing an exceptionally high number of 570,292 reads distributed among 97 OTUs. One replicate of the DNase treated stream water eRNA sample of the Grüner Bach did not contain any reads after bioinformatic quality filtering. However, after merging both replicates the other replicate contained enough reads for subsequent analysis of the sample.

In total, we retained 1,498 OTUs and 682 unique species after retaining only OTUs that had a similarity of at least 90% to a reference sequence and subtracting the reads of the negative controls (see Suppl. material 5). The MH eDNA samples of the Boye after incubation contained only 15 OTUs, therefore, we removed the samples from the Boye from all statistical tests in the results section. After subsampling the MH and NSE samples, we observed a similar proportion of shared species between sampling approaches (not subsampled: 63%; subsampled: 68%) but also of exclusive species (not subsampled: 4% MH-exclusive, 32% NSE-exclusive; subsampled: 6% MH-exclusive, 25% NSE-exclusive (Suppl. material 1: fig. S4)). Therefore, we used the not subsampled dataset for all comparisons.

Comparison of local signal in stream water eRNA versus eDNA samples

In order to test hypothesis 1, species lists obtained via eDNA and eRNA metabarcoding of stream water were compared to those obtained from MH and NSE bulk DNA metabarcoding (local signal references). We found a 13.65% higher proportion of shared species for eDNA stream water and bulk samples than for eRNA stream water and bulk samples (Fig. 3). The read proportion of shared species between bulk samples and stream water eDNA samples was also higher (75–80%) than in comparison to the stream water eRNA samples (50–70%) (Fig. 3).

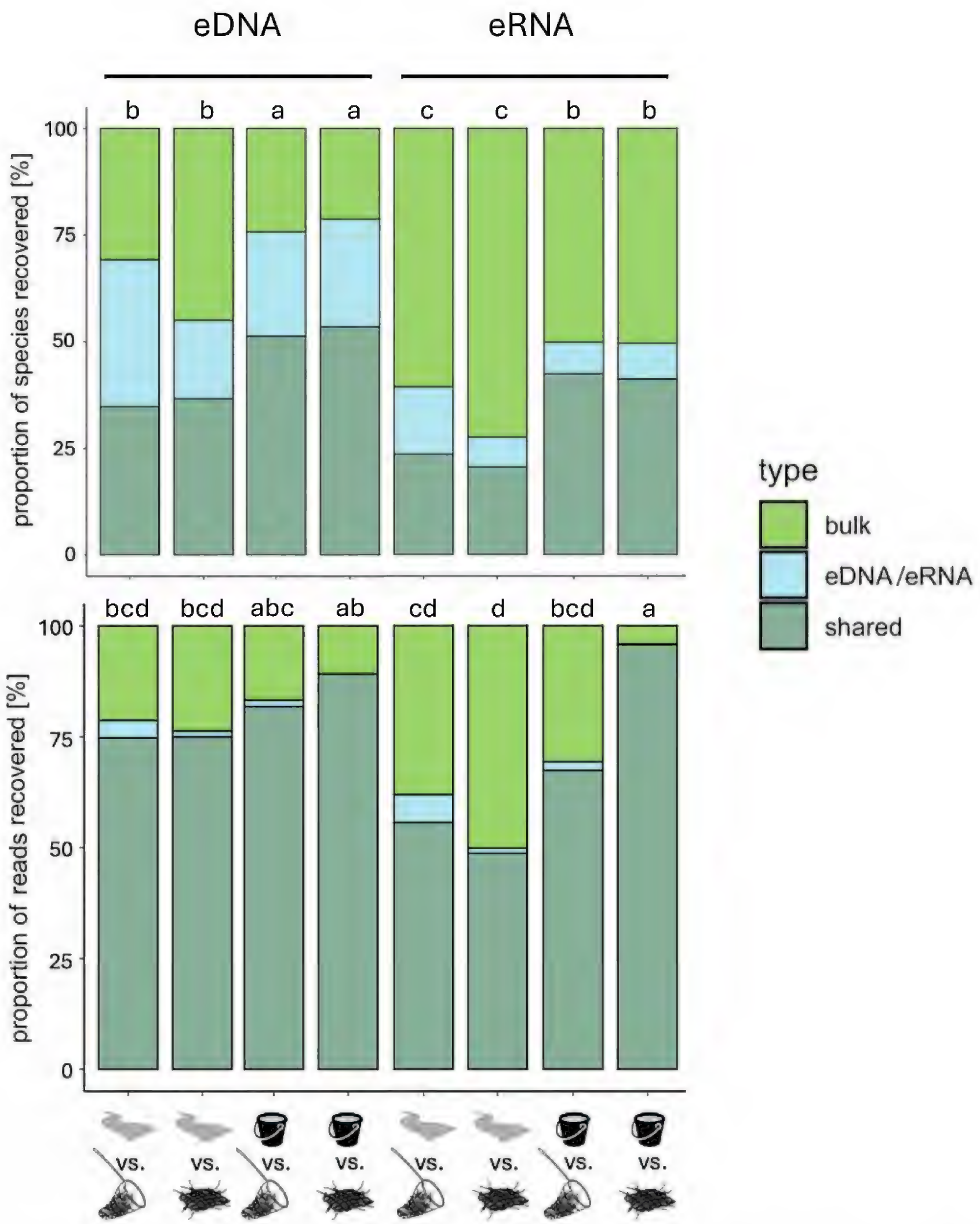

Figure 3. Proportion of species (upper bars) and reads (lower bars) recovered with the different sampling approaches averaged across all sampled streams (excluding Boye) with eDNA (left) and eRNA metabarcoding (right). Total proportion of detected species/reads for each category: bulk-exclusive (light green), eDNA/eRNA-exclusive (light blue) and shared (dark green). Stream icon showing direct stream water sampling and bucket icon the incubation sampling approach. MH bulk samples are presented with a net icon, NSE bulk samples with an icon showing an NSE, MH and NSE eDNA/eRNA samples with a bucket icon and stream water eDNA/eRNA samples with a stream icon. Results of the pairwise t-tests for species (upper bars) and pairwise Wilcoxon tests for reads (lower bars) are displayed above each bar as letters. Significance was calculated between the different sampling approaches including both eDNA and eRNA. Sampling approaches sharing a letter are not significantly different from each other, while sampling approaches with different letters differ significantly from each other based on the results of the pairwise t-tests and pairwise Wilcoxon tests. The letters are arranged in order of increasing mean, starting with “a” as the highest value to the lowest. See Suppl. material 1: fig. S5 for a schematic depiction of the sampling approaches that were compared to test the different hypotheses.

Comparison of local signal in incubated water versus stream water samples

To verify the first part of our second hypothesis (hypothesis 2a), we tested if the overlap between species and OTU lists obtained using eDNA/eRNA metabarcoding after incubation and bulk DNA metabarcoding would be higher than the overlap between stream water eDNA/eRNA metabarcoding and bulk DNA metabarcoding. The proportion of species and OTUs detected using both sample types (eDNA/eRNA and bulk) increased significantly (eDNA: 16.67%; eRNA: 19.74%) after incubation compared with direct stream water sampling (ANOVA: eDNA: F-value = 23.2, p-value < 0.001, numerator degrees of freedom (ndf) = 3, denominator degrees of freedom (ddf) = 24 (species); F-value = 26.72, p-value < 0.001, ndf = 3, ddf = 24 (OTUs); eRNA: F-value = 921.1, p-value < 0.001, ndf = 3, ddf = 24 (species); F-value = 11.65, p-value < 0.04, ndf = 3, ddf = 24 (OTUs)) (Fig. 3, Suppl. material 1: fig. S6, tables S2–S5). In addition, using eRNA metabarcoding of incubated water samples detected a higher proportion of species shared with the bulk samples than the stream water eDNA samples (Fig. 3). Between the two incubation sampling approaches, MH and NSE, we detected no significant difference in the number of shared species or reads recovered in the water samples. However, for the incubated eRNA water samples of the NSE sampling approach, the proportion of shared reads was significantly higher than for the other eRNA water samples (Fig. 3, chi-square = 15.61, p-value = 0.001). Since the stream water eDNA and incubated eDNA water samples yielded higher species and OTU numbers and showed higher similarity to the species and OTUs detected in the bulk samples, we focused our results in the following sections on the eDNA samples and excluded the eRNA samples from subsequent NMDS analyses and the comparison of the two incubation sampling approaches MH and NSE.

Furthermore, NMDS analysis revealed species communities to be more similar between eDNA and bulk samples after the incubation than between direct eDNA stream water samples and bulk samples (Fig. 4). Regardless of the stream sampled, the eDNA and bulk samples of the MH and NSE sampling approaches clustered closely together, whereas the stream water samples showed higher dissimilarity among each other and to the bulk samples. Nevertheless, samples belonging to the same stream showed high similarity to each other and clustered closely together (Fig. 4). The Felderbach stream samples, especially the second sampling spot (FB2), displayed high dissimilarities among samples, whereas samples of the two incubation sampling approaches clustered more closely together. Based on the ANOSIM test, the species community composition of the three sampling approaches (stream water sampling, MH incubation and NSE incubation) were significantly different from each other, though they showed some overlap based on the R value (p-value < 0.001, R-value = 0.34). The pairwise comparisons showed that while the samples of the incubation sampling approaches (MH and NSE) did not differ from each other (adjusted p-value = 0.195, R^2 = 0.024), they both differed significantly from the samples taken directly from the stream (adjusted p-value = 0.003, R^2 = 0.097 (stream:MH); adjusted p-value = 0.003, R^2 = 0.088 (stream:NSE)). The species community composition of the four streams also differed significantly from each other showing a higher dissimilarity than between sampling approaches (p-value < 0.001, R-value = 0.68).

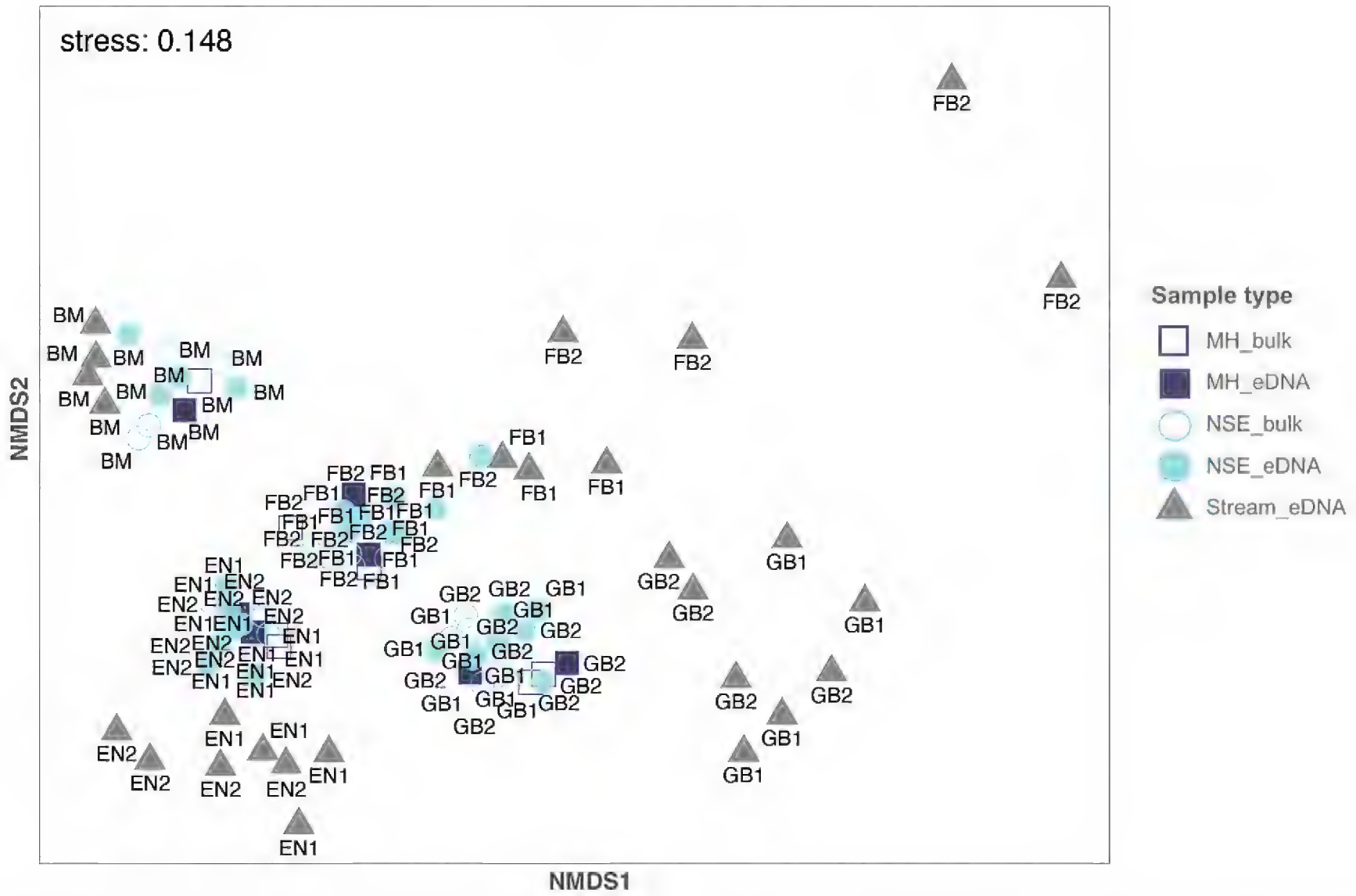

Figure 4. Non-metric multidimensional scaling (NMDS) based on Jaccard's distance for macroinvertebrate species. Shown are samples from the MH incubation sampling approach (dark blue squares), the NSE incubation sampling approach (light blue circles) and the stream water sampling (gray triangles). Filled symbols represent eDNA water samples and empty symbols bulk samples. BM = Borbecker Mühlenbach (tributary of Emscher), EN1 = Ennepe sampling spot 1, EN2 = Ennepe sampling spot 2, FB1 = Felderbach sampling spot 1, FB2 = Felderbach sampling spot 2, GB1 = Grüner Bach sampling spot 1, GB2 = Grüner Bach sampling spot 2.

A high proportion of species was exclusively detected in the stream water samples but not in the bulk samples of either sampling approach (MH and NSE). Here, the proportion of species detected in the stream water samples, which were not detected in the bulk samples (on average 44% compared to MH bulk, 34% compared to NSE bulk) was almost twice as high as the proportion found in the incubated eDNA water samples (on average 19% MH sampling approach, 25% NSE sampling approach) (Suppl. material 1: tables S2–S5). However, most OTUs and species were shared between all three sampling approaches (stream water sampling, MH incubation (eDNA and bulk) and NSE incubation (eDNA and bulk)) in general but also across macroinvertebrate orders (Suppl. material 1: tables S6–S9). Most species and OTUs exclusively found in the stream water samples belonged to groups Diptera, Annelida and Nematoda. Species that were more commonly found in MH and NSE samples including the eDNA, belonged to orders Ephemeroptera and Trichoptera. Here, we found 15 OTUs and species that were not found in the stream water samples. Further, for order Amphipoda, the stream water samples did not detect the amphipod *Gammarus fossarum*, which was detected in the eDNA and bulk samples of both incubation sampling approaches (Suppl. material 1: tables S6–S9). However, over 20 OTUs were detected for the order Coleoptera in the bulk samples of both incubation sampling approaches which were not detected in any of the eDNA samples. These 20 OTUs corresponded to only three species (Suppl. material 1: tables S6–S9). In contrast, for the group Bivalvia, only one species was detected in the bulk samples of both

incubation sampling approaches, while the stream water samples detected one species, *Euglesa casertana*, being exclusive to the stream water samples.

Shared reads were distributed more evenly between eDNA and bulk samples for the incubation sampling approaches, especially for the NSE approach, than for the stream water samples (Suppl. material 1: fig. S7). Likewise, read proportion distribution among macroinvertebrate groups was similar for both incubation sampling approaches and differed from the proportions obtained from the stream water samples (Suppl. material 1: fig. S8). Read proportions, especially for more sclerotized groups like Amphipoda and Coleoptera but also for other groups like Plecoptera and Megaloptera were higher for the bulk samples than the water samples (incubation and stream), whereas the read proportions for planktonic groups (like daphnids and copepods) were highest for the stream water samples (Suppl. material 1: fig. S8).

Comparison of local signal in incubated water eRNA versus eDNA samples

For the second part of our second hypothesis (hypothesis 2b), we tested if the overlap between species lists obtained from eRNA from incubated water samples and MH and NSE bulk samples is higher than the overlap between eDNA from incubated water samples and MH and NSE bulk samples. The incubation sampling approach using eDNA detected 10% more species shared between eDNA and bulk than the incubation sampling approach using eRNA. Further, over 20% more species were detected in the bulk samples with the incubation sampling approach using eDNA than with the incubation sampling approach using eRNA (Fig. 3, Suppl. material 1: fig. S9). Although for some samples, e.g., for the Boye, using eRNA improved detection of shared species (Suppl. material 1: tables S2–S5).

Comparison of multi-habitat sampling vs natural substrate exposure

In order to test hypothesis 3, we compared species lists obtained via eDNA metabarcoding and bulk DNA metabarcoding of the two sampling approaches, MH and NSE. We detected 20% more species with the NSE sampling approach with both eDNA and bulk DNA metabarcoding (Fig. 5, Suppl. material 1: figs S10, S11). Although the majority of species (63%) were shared between both sampling approaches (for eDNA and bulk), only 26 species were exclusive in the MH samples, while 94% were also detected in the NSE samples. The NSE sampling approach revealed higher species numbers for nearly all macroinvertebrate groups, except for Megaloptera, Cnidaria, and Acari with equal numbers for the NSE and MH samples (eDNA and bulk). For the order Hemiptera, more species were detected using the MH sampling approach, and one amphipod species was exclusively detected in the bulk samples of the MH sampling approach, the groundwater amphipod *Niphargus aquilex*, whereas no amphipod species was exclusive to the NSE sampling approach (Fig. 5, Suppl. material 1: figs S10, S11). Species of the orders Plecoptera, Isopoda, Ephemeroptera and Bivalvia were exclusively detected in the NSE samples but not in the MH samples (eDNA and bulk); and Odonata was the only order where all species were exclusively found using the NSE sampling approach (eDNA and bulk) (Fig. 5). For the NSEs, the proportion of species detected in both, in the incubated eDNA water and in

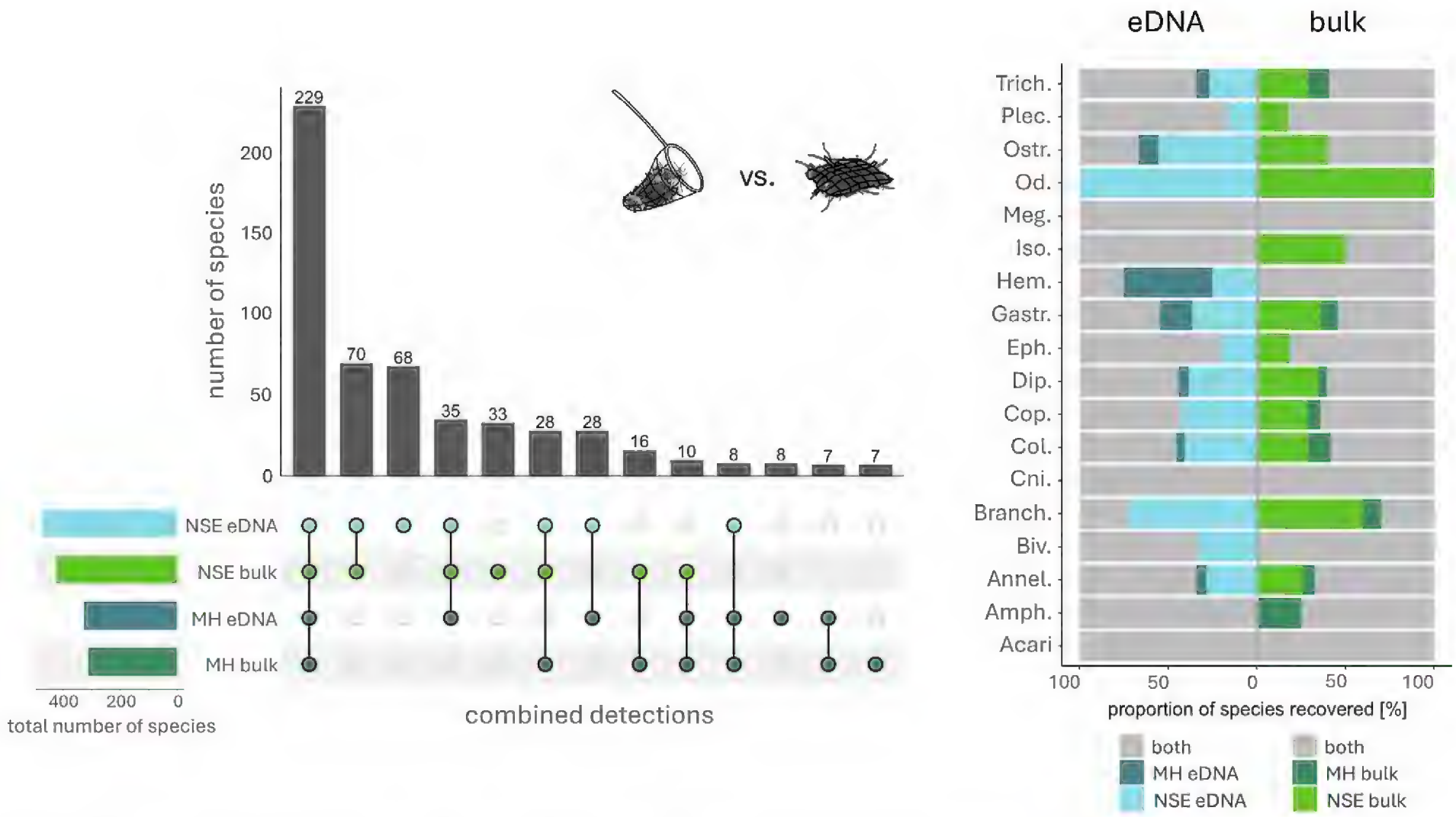

Figure 5. Comparison of the species detected via metabarcoding of multi-habitat (MH) and natural substrate exposure (NSE) bulk samples and eDNA from incubated water samples of these. Left: species shared between the two incubation sampling approaches (MH: darker colors; NSE: lighter colors) and between the eDNA (blue) and bulk samples (green) and those that were exclusively found. Filled circles per row indicate combined detections. Right: proportion of species recovered with both incubation sampling approaches (grey) and with only one approach for the eDNA (left; MH: dark blue; NSE: light blue) and bulk (right; MH: dark green; NSE: light green) samples per macroinvertebrate group (Trich. = Trichoptera; Plec. = Plecoptera; Ostr. = Ostracoda; Od. = Odonata; Meg. = Megaloptera; Iso. = Isopoda; Hem. = Hemiptera; Gastr. = Gastropoda; Eph. = Ephemeroptera; Dip. = Diptera; Cop. = Copepoda; Col. = Coleoptera; Cni. = Cnidaria; Branch. = Branchiopoda; Biv. = Bivalvia; Annel. = Annelida; Amph. = Amphipoda; Acari).

the organismal bulk samples, was on average 43% and ranged from 34% (Boye) to 52% (EN2) (Suppl. material 1: fig. S12, table S10). The average overlap only increased to 53%, when the species found by the four NSEs were merged per sampling site, which was close to the average overlap detected using the MH sampling approach (51%) (Suppl. material 1: tables S11, S12). In addition, the four NSEs per site shared on average 24% (eDNA) and 26% (bulk) of the species, with 10% (for eDNA and bulk) being found exclusively in just one NSE (Suppl. material 1: fig. S13). Both substrate types showed a comparable species richness (504 (wood-leaf) and 484 (gravel)) and shared most of the detected species (75%).

Discussion

Our results demonstrate that among the three strategies introduced in the beginning of this study, utilising natural substrate exposures (NSEs) followed by incubation prior to eDNA sampling yielded the highest similarity to species lists generated from bulk metabarcoding of organisms. Although the incubation strategy using multi-habitat (MH) kicknet sampling also increased the overlap between species lists obtained by incubated eDNA water samples and organismal bulk samples in comparison to stream water eDNA and eRNA samples, it detected 20% less species than the incubation strategy using NSEs. Therefore, in the given setting of

the study, using NSEs for the collection of macroinvertebrates followed by incubation represents the most promising strategy for local and standardised eDNA metabarcoding assessments. However, each strategy has distinct advantages and disadvantages with regard to the sampling effort and the invasiveness of the sampling process to animals and the streambed, which are further discussed below.

Does eRNA metabarcoding improve the detection of local species? (hypothesis 1, strategy 1)

We hypothesised that the overlap of species detected by metabarcoding from both bulk samples (local community) and eRNA samples of stream water (strategy 1) is higher than the overlap of species detected by the locally collected bulk samples and eDNA of stream water. In contrast to our hypothesis, eDNA metabarcoding reflected the local community better than eRNA metabarcoding. We expected that the species lists obtained using eRNA metabarcoding would be more similar to the local community than the species lists obtained via eDNA metabarcoding, because RNA has a higher production rate than DNA and therefore, accumulates faster in the water column (Von Ammon et al. 2019; Wood et al. 2020). In addition, RNA is reported to often decay faster than DNA (Eigner et al. 1961; Mengoni et al. 2005), thereby reducing the regional signal of the eRNA at a higher rate than the signal of the regional eDNA. When analysing stream water, we did, in fact, detect a lower amount of eRNA- than eDNA-exclusive species. However, we also detected fewer local species (assessed via the organismal bulk samples) in the eRNA stream water samples than in the eDNA stream water samples. These results suggest that eRNA metabarcoding of the stream water samples detected in general fewer species than eDNA metabarcoding, independent of the assumed origin of the DNA (regional or local). Therefore, small or elusive species were probably even less likely detected with eRNA metabarcoding than with eDNA metabarcoding. A potential source for the fast eRNA decay of our samples was the sampling and sample storage in the field. Although we filtered the water samples directly after sampling and stored them on ice in the field, the sampling process took several hours, while the samples were cooled but not frozen. Upon returning from the field, the samples were immediately stored in a laboratory freezer at -80 °C. The stability of eRNA molecules in the field cannot be confirmed, as it is unclear whether the proportion was already lower in the stream or if degradation occurred during handling or transport. However, research shows that eRNA can remain stable in the field for up to 24 hours (Scriver et al. 2024). Our results, however, agree with findings of another study that found significantly lower richness values for COI-based metabarcoding of eRNA compared to eDNA for stream invertebrates (Macher et al. 2024).

Does incubation of locally collected samples improve the signal of local species in eDNA metabarcoding? (hypothesis 2a, strategies 2 and 3)

According to the first part of our second hypothesis, we expected that the overlap of species lists from metabarcoding of bulk samples and eDNA/eRNA from incubated water samples increases with incubation time regardless of sampling approach (MH samples, strategy 2, or NSE samples, strategy 3). In accordance with our hypothesis, we detected a 17%/20% higher overlap between species

lists obtained from bulk and eDNA/eRNA metabarcoding after incubation than between bulk samples and eDNA/eRNA samples from the stream water samples. Similar to the results of Sander et al. (2025), we detected a high overlap of > 50% between the species lists of the bulk samples and the incubated eDNA water samples. After merging all species lists from all sampling spots at a site, the overlap of species detected in the bulk samples and incubated eDNA water samples increased to 67%, while the overlap between species detected by the bulk samples and stream water samples reached 53%. This overlap for the direct stream water samples is similar to the ones reported by studies when either a certain taxonomic group such as EPT (Macher et al. 2018) or a higher taxonomic level was examined such as family (Deiner et al. 2017) or genus (Mächler et al. 2019). However, the species overlap was still significantly lower than the overlap for the incubation sampling approach. We still found a relatively high overlap (~35%) between species lists obtained from organismal bulk samples and stream water samples when considering samples from each sampling spot individually in comparison to other studies reporting an overlap of < 20% when comparing species recovery rates between bulk samples and stream water samples (e.g., Hajibabaei et al. 2019; Gleason et al. 2021; Pereira da Conceicoa et al. 2021). This can be attributed to the fact that the water samples were collected from areas directly below the spots where the NSEs were attached. The smaller size of the sampled streams and low water flow may have constrained the downstream movement of eDNA and enhanced the detection of species found in the immediate area (Shogren et al. 2018; Curtis et al. 2021).

The stream water eDNA samples exhibited a relatively high proportion of exclusive species, particularly in comparison to the bulk samples. Additionally, these exclusively detected species were not detected in the incubated eDNA water samples. However, metabarcoding of the stream water samples of the sampling spot FB2 detected an exceptionally low number of species and a low number of species exclusive to eDNA. This was also observed, although less pronounced, in the other water samples of the sampling spot FB2. We do not know what caused these dissimilarities between sampling spots, as both sampling spots of the Felderbach were in close proximity (750 m) and therefore, had similar stream characteristics. However, it should be noted that not all species that were exclusive to the stream water samples should be attributed to a regional signal (i.e., DNA from not locally present organisms). The reason for this is that the MH and NSE sampling approaches do not capture every species present in the local reach of the stream, but rather a subset of the total community. Water samples can provide a more comprehensive and holistic representation of the entire ecosystem, from local and regional sources (Deiner et al. 2016; Stat et al. 2017; Macher et al. 2021a). Interestingly, when comparing the species lists of the stream water eDNA samples and the organismal bulk samples of the MH sampling approach, we found that some of the species that were exclusively detected in the stream water eDNA samples were also detected in the organismal samples of the NSE sampling approach. This suggests that the DNA of these species was not necessarily originating from further upstream but from local occurring species that were not collected via the MH sampling approach (but the NSEs), resulting in the detection of false negatives. The differences in the number of species detected between the two incubation sampling approaches (MH and NSE) was the subject of our last hypothesis.

Does eRNA metabarcoding of incubated water of locally collected samples further improve the signal of local species in comparison to eDNA metabarcoding? (hypothesis 2b, strategies 1, 2 and 3)

As another part of our second hypothesis, we predicted that species lists obtained via eRNA metabarcoding of the incubated water show a slightly greater overlap with the locally collected MH and NSE bulk samples than eDNA metabarcoding of the incubated water. In contrast to our prediction taxa lists obtained by eRNA metabarcoding of incubated water were less similar to the locally collected samples (MH and NSE DNA bulk samples) than eDNA metabarcoding. As already mentioned, when discussing hypothesis 1, the results suggest that eRNA metabarcoding using COI for invertebrates detects fewer species than eDNA metabarcoding, independent of the assumed origin of the DNA (regional or local; see Macher et al. 2024). However, when incubating the animals before taking a water sample, the proportion of species detected in both the eRNA water samples and organismal bulk samples was twice as high and the number of species being exclusive to the eRNA water samples decreased in comparison to the direct eRNA stream water samples. This indicates that the eRNA was likely already less abundant prior to sampling the streams and that minimal decay occurring during the sampling and transportation process was not the main reason for the low number of species detected in the stream water samples using eRNA metabarcoding (Scriver et al. 2024). The study conducted by Littlefair et al. (2022) demonstrated comparable outcomes in species detection between eRNA and eDNA metabarcoding, using comparable field methodologies to those employed in eDNA sampling. These results suggest that the effect of the molecule (eRNA or eDNA) used for metabarcoding on the detection of species captured with both water samples and organismal bulk samples is lower than the effect of incubation, which we investigated further in the context of the first part of our second hypothesis. Thus, our study does not provide strong arguments in favour of using eRNA to improve the local signal for bioassessments.

Advantages of natural substrate exposures and multi-habitat sampling (hypothesis 3, strategies 2 and 3)

In our last hypothesis, we predicted that MH kicknet sampling, recovers more species than NSE sampling because of the broader habitat range sampled and the higher surface area of the streambed being sampled per site using the multi-habitat sampling approach of strategy 2 (NSE: 0.360 m^2; MH: 0.625 m^2). Contrary to our prediction, 20% more species were detected in the NSEs, regardless of macroinvertebrate order than in the MH samples. Further, 94% of the species detected by MH sampling were also detected by the NSE sampling. Additionally, to the expected higher number of specimens agitated by kicknet sampling, we expected eDNA retained in the sediment to be released, resulting in an even higher detected species richness. One potential explanation for the higher number of species found in the NSEs is that the artificial substrates offer a richer, more spatially condensed array of food resources, including foliage, wood and biofilm. This likely increased the number of macroinvertebrate species at this resource-rich and diverse spot. Additionally, the nets and

their anchoring in the streambed provide protection and a stable habitat that is resistant to strong currents. However, when assessing the species shared between eDNA and bulk samples for only one NSE per site, the proportion of shared species was reduced by 10% in comparison to the overlap achieved with all four NSEs combined. Furthermore, the four NSEs per site shared only 20–30% of their species. Besides a simple density-dependent effect resulting in insufficient space for members of all species on one NSE, this is likely due to the different habitats covered (wood-leaf and gravel) and the placement of the substrates at both low currents (pools) and high currents (riffles), leading to the attraction of different species depending on the substrate type and placement in the stream (Scullion et al. 1982; Logan and Brooker 1983; Cummins 2016). Although we detected more species in the NSE samples than in the MH samples, the composition of the species communities and species identified were similar. Only the order Odonata was exclusively detected in the NSE samples. The four species (*Aeshna cyanea*, *Aeshna mixta*, *Enallagma cyathigerum*, *Chalcolestes viridis*) avoid currents and prefer standing waters (Schmedtje and Colling 1996; Tachet et al. 2010; Schmidt-Kloiber and Hering 2015, 2024; Dijkstra et al. 2024). Consequently, all the species were detected solely in those NSEs that were placed at the spots with low flow velocity, which likely attracted the dragonfly larvae, resulting in their aggregation at these NSEs.

When applying MH kicknet sampling, we mainly focused on the microhabitats of the stream and did not pay particular attention in sampling evenly between pools and riffles, which might have been a factor influencing the non-detection of Odonata and other species in the MH samples. In contrast, the amphipod *Niphargus aquilex* was exclusively present in the MH samples. This species inhabits groundwater habitats and was therefore only captured during the sampling process using the kicknet approach, which is a more invasive sampling approach that agitates the streambed and could therefore also catch animals that are living in the ground close to the streambed surface. Consequently, the choice of sampling approach should be carefully considered, taking into account the animals that are captured using different approaches and the objective of the study.

Potential and possible obstacles for the application of the three strategies for biomonitoring

While the incubation strategy using NSEs yielded the most comprehensive results, it is important to acknowledge the distinct advantages and disadvantages associated with each of the three strategies proposed in this study in terms of their potential applicability for biomonitoring purposes. With the first strategy, we tested the potential of eRNA metabarcoding of stream water as a means to more locally assess stream communities. However, sampling and subsequent analysis of the samples must be done as fast and sterile as possible to avoid degradation of the more unstable eRNA molecules through temperature, microbial activity, pH or RNA-degrading enzymes (Li and Breaker 1999; Zulkefli et al. 2019; Mauvisseau et al. 2022; Jo et al. 2023). To prevent this, it is important to immediately filter the samples and to store them on ice in the field to decelerate the degradation process. To counteract the degradation of eRNA on the filters, RNA stabilisation reagents such as RNA*later* (Miyata et al. 2021; Littlefair et al. 2022) may be employed. The addition of RNA*later* has been demonstrated to maintain

RNA concentrations in a stable state for up to one month at room temperature (Gorokhova 2005). However, these reagents are costly, and the additional expense of the reagents required for DNase digestion and reverse transcription can significantly increase the cost of analysing a single sample (Macher et al. 2024). Thus, a number of factors must be taken into account when using eRNA metabarcoding, including the type of RNA (mRNA vs. rRNA), the source of RNA (nuclear vs. mitochondrial) and the gene region to be employed (Pikó and Taylor 1987; Sidova et al. 2015; Marshall et al. 2021; Jo et al. 2023).

Interestingly, this study found eDNA metabarcoding of water samples (stream water and incubated water) to generate more similar results to metabarcoding of the locally sampled organisms than eRNA metabarcoding. This result holds true for both direct stream water sample comparisons as well as for the incubated water samples. However, eRNA metabarcoding is still in its infancy, and in streams decay and transport from upstream sources must not necessarily be lower given intracellular transport possibilities of eRNA particles (Jo 2024). Further research is needed to explicitly uncover eRNA dynamics. For sure, the analysis of eRNA offers significant promise for the future, particularly in assessing the physiological status and health of individuals or the ecological stress response following a local impact. Therefore, in the future eRNA analysis might function as a predictive tool for the detection of changes on the molecular level before eDNA analysis or traditional biomonitoring could detect changes in species composition.

A key finding was the use of eDNA data derived from incubation of locally collected organisms using MH sampling (strategy 2) and NSEs (strategy 3). In this context, NSEs have the benefit of the gentle removal of the animals from the stream with little work safety requirements, because the stream does not need to be entered, and minimal harm for the sampled organisms compared to the kicknet sampling of the MH strategy. However, the effort involved in kicknet sampling, and the preceding mapping of the streams' microhabitats is lower in comparison, as only a net is used for sampling and no preparatory work is required as in the NSE strategy (collecting substrate, preparing NSEs, deposition and collection of NSEs) and the net can quickly be reused. Additionally, the deposition and anchoring of the NSEs to the streambed is more challenging in larger streams with high currents, which was one reason why the Emscher was not sampled in this study, but rather its smaller tributaries. The use of NSEs has the benefit of a more standardised selection of substrates (De Pauw et al. 1986), similar to the already standardized use of ARMS (Artificial reef monitoring substrates) in marine environments (Ransome et al. 2017). However, this potential is limited in streams with diverse habitats or where the replication of stream substrates is not feasible, particularly those with sandy substrates that allow animals to bury themselves. Here the use of multi-habitat kicknet sampling may provide a more accurate representation of the stream's microhabitats. This may also have been a limitation when sampling the Boye, a small sand-dominated lowland river, as the number of species identified in the MH and NSE bulk samples was comparable for the Boye, yet the NSE bulk samples showed a greater increase in species numbers relative to the MH bulk samples for the other studied streams. Furthermore, for the Boye a considerable number of species were exclusively present in the incubated eDNA water samples and absent in the bulk samples. For the identified advantages of NSEs, further verification is required to confirm this result in other stream types. Interestingly, the stream area that was

covered by the NSE sampling approach (0.360 m^2) constituted approximately half of the area sampled using the MH approach (0.625 m^2), which was already reduced by half, due to the smaller size of the studied streams and their homogeneous microhabitats, in comparison to the standardised area sampled in accordance with the WFD (Meier et al. 2006). To further enhance the number of species collected using the NSE sampling approach, it would be recommended to increase the number of NSEs introduced into the stream, thereby increasing the sampled area. Lastly it should be considered to test if an increase of the incubation time would result in a higher overlap of species detected in both the bulk and the incubated eDNA water samples or if a reduction of the incubation time would be sufficient to yield comparable results. The preceding study demonstrated an immediate high degree of overlap between bulk and incubated eDNA water samples following the transfer of the animals to the incubation medium, with no significant increase over time (Sander et al. 2025). Consequently, the time required for the incubation of the animals may be reduced, thereby allowing for an increase in the number of sample sites sampled on a sampling campaign.

Conclusions

Our study demonstrates that the most effective strategy to enhance the detection of the local stream community via water samples involves briefly incubating collected organisms in water, followed by the analysis of the enriched eDNA. Species lists generated after a prior incubation of the collected animals show a higher overlap with the species lists derived from the organismal bulk samples than those obtained via direct eDNA and eRNA metabarcoding of stream water samples. Natural substrate exposures provided higher species numbers for both the bulk samples and the incubated eDNA samples than classical kicknet multi-habitat sampling. Thus, this less invasive avenue should be considered further for bioassessments despite the higher workload prior to sampling. Further research is needed to determine the effectiveness of NSEs in other stream types before potential uptake into standardised biomonitoring programs.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

Conceptualization: MS, FL, AJB, MW, DB; Formal analysis: MS, DB, DB; Investigation: MS, DB; Methodology: MS, DB; Supervision: FL, AJB, MW; Writing - review and editing: MS, FL, AJB, MW,DB, DB. Funding acquisition: FL, MS. Project administration: FL. Resources: FL. Supervision: FL, AJB, MW. Visualization: MS. Writing - original draft: MS.

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Data availability

All raw reads are accessible via the European Nucleotide Archive (ENA) under accession number PRJEB85525.

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Supplementary material 1

Supplementary figures and tables

Authors: Mandy Sander, Arne J. Beermann, Denis Brömmling, Dominik Buchner, Martina Weiss, Florian Leese

Data type: docx

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Supplementary material 2

Stream habitat mapping

Authors: Mandy Sander, Arne J. Beermann, Denis Brömmling, Dominik Buchner, Martina Weiss, Florian Leese

Data type: xlsx

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Supplementary material 3

Statistical output

Authors: Mandy Sander, Arne J. Beermann, Denis Brömmling, Dominik Buchner, Martina Weiss, Florian Leese

Data type: xlsx

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Supplementary material 4

Unfiltered OTU table, sample ID and description

Authors: Mandy Sander, Arne J. Beermann, Denis Brömmling, Dominik Buchner, Martina Weiss, Florian Leese

Data type: zip

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Supplementary material 5

Filtered OTU and species tables

Authors: Mandy Sander, Arne J. Beermann, Denis Brömmling, Dominik Buchner, Martina Weiss, Florian Leese

Data type: xlsx

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